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Controlling embryonic stem cell proliferation and pluripotency: the role of phosphoinositide 3-kinase- and glycogen synthase kinase-3-dependent signaling

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Abbreviations: ERas, ES cell-expressed Ras; ERK, extracellular regulated kinase; ESC, embryonic stem cell; GSK-3, glycogen synthase kinase-3; LIF, leukaemia inhibitor factor; MEK, MAP and ERK kinase; mTOR mammalian target of rapamycin; PI, phosphoinositide; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; Stat3, signal transducer and activator of transcription 3

Key words: embryonic stem cells; pluripotency; PI3K; GSK-3; self-renewal; proliferation; cell cycle

Abstract

Embryonic stem cells (ESCs) are derived from the inner cell mass of preimplantation embryos and are pluripotent, meaning they can differentiate into all cells that make up the adult organism. This property of pluripotency makes ESCs attractive as a model system for studying early development and for the generation of specific cell types for use in regenerative medicine and drug screening. In order to harness their potential, the molecular mechanisms regulating ESC pluripotency, proliferation and differentiation (i.e. cell fate) need to be understood such that pluripotency can be maintained during expansion, while differentiation to specific lineages can be induced accurately when required. This review will focus on the potential roles that phosphoinositide 3-kinase (PI3K) and glycogen synthase kinase 3 (GSK-3)-dependent signaling play in coordination and integration of mouse ESC pluripotency and proliferation and contrast this with our understanding of their functions in human ESCs.

Control of embryonic stem cell fate: an overview

Embryonic stem cells are derived from early pre-implantation embryos and when cultured appropriately can be maintained in a proliferative, self-renewing, pluripotent state almost indefinitely. Pluripotency is the ability to differentiate into all cells found in an adult organism, while self-renewal describes the generation of a daughter stem cell from its mother. In the case of ESCs, self-renewal occurs symmetrically, such that when an undifferentiated ESC divides and pluripotency is maintained, both its progeny will be undifferentiated [1]. Thus, although not commonly described as such, self-renewal of ESCs requires simultaneous control of both the cell cycle (proliferation) and maintenance of pluripotency. It is important to emphasize that self-renewal is NOT the same as proliferation [2, 3] because the latter can occur when pluripotency is not maintained.

Over the past 5-10 years our understanding of the molecular components involved in maintaining pluripotency of mouse ESCs has increased dramatically, from a simple 'prelude' where Signal transducer and activator of transcription (Stat3) activation by Leukaemia inhibitor factor (LIF) was all that seemed necessary, to a complex 'symphony' where extrinsic factors, intracellular signals, transcription factors, epigenetic regulators and microRNAs have all been implicated [4]. Three transcription factors, Oct4, Sox2 and Nanog, are considered the 'master' regulators of ESC pluripotency and were initially proposed to form a self-sustaining gene regulatory network (reviewed in [5]). However, revision of this model is now necessary to accommodate recent findings, namely that ESC populations are not homogeneous [6, 7] and indeed, within populations of ESCs, expression of Nanog [8] *Essrb* [9] and *Rex1* [10] have all been shown to be both heterogeneous and dynamic. Despite our progress in understanding the factors that influence pluripotency there remains a significant gap in our knowledge regarding the molecular mechanisms responsible for the coordination and integration of ESC proliferation and pluripotency [2, 3].

The ESC cell cycle

Mouse ESCs proliferate rapidly in culture and display unique cell cycle kinetics, distinct from those of somatic cells, dividing approximately every 11-16h and exhibiting a shortened G1 phase [11, 12]. Throughout the mouse ESC cell cycle it has been reported that cyclin levels remain relatively stable [12], cyclinA/E:cyclin-dependent kinase 2 (Cdk2) complexes are active and the Retinoblastoma protein remains hyperphosphorylated [11, 12]. In addition, cell cycle progression appears to be insensitive to inhibition by p16^{INK4a} [13] and neither do mouse ESCs arrest following DNA damage [14]. By contrast, it has been reported that levels of cyclins D1, D2 and D3 do vary throughout the cell cycle of human ESCs and knock-down of CDK2 leads to G1 arrest and differentiation to extraembryonic lineages [15]. Recently, in human ESCs pluripotency regulators

have been linked directly with control of the cell cycle. It has been demonstrated that mir302 miRNAs are regulated by OCT3/4 and SOX2, that mir302a targets cell cycle regulators and that inhibition of mir302a results in increased cyclin D1 levels and increased numbers of hESCs in G1 [16]. In mouse ESCs it has been reported that the mir290 cluster of miRNAs regulate G1/S-phase transition by controlling levels of p21^{Cip} and p27^{Kip1} and are important for promoting rapid proliferation [17]. These results are somewhat at odds with earlier studies that had suggested little role for these cyclin-dependent kinase inhibitors in the control of the mouse ESC cell cycle. Thus, while control of the cell cycle may vary between mouse and human ESCs, a number of important questions relating to this regulation remain unanswered.

Phosphoinositide 3-Kinase (PI3K) signaling and ESC biology

PI3Ks are a family of lipid kinases, comprising 3 sub-classes [18, 19]. Class I_A PI3Ks are heterodimers comprising a regulatory subunit (3 genes, 5 isoforms, p85 α , p55 α , p50 α , p85 β & p55 γ) and a catalytic subunit (3 genes and 3 isoforms: p110 α , β and δ). The primary product of class I PI3K activity is the phosphoinositide PI(3,4,5)P₃ which recruits pleckstrin homology domain-containing proteins to the plasma membrane, facilitating activation of a range of downstream signalling cascades [20]. Functionally, PI3Ks have been implicated in a wide array of physiological processes including proliferation, development, growth and migration [20]. Of particular interest in relation to ESCs is the report that ablation of p110 β leads to lethality at the pre-implantation stage, suggesting a key role for PI3Ks during early mouse embryogenesis [21]. Studies conducted over the past few years have suggested roles for PI3Ks in both control of ESC cell cycle, proliferation and also in maintenance of pluripotency.

Involvement of PI3Ks in control of ESC proliferation The first suggestion that PI3Ks were involved in control of ESC proliferation came from the observation that mouse ESCs lacking the PTEN tumour suppressor gene (a negative regulator of PI3K signaling) exhibited enhanced proliferation and diminished dependence on serum [22]. Subsequently, incubation of murine ESCs with high doses of the broad selectivity PI3K inhibitor, LY294002, decreased proliferation and led to cells accumulating in G1 [23]. Eras, a constitutively active member of the Ras family of small GTPases, is expressed specifically in mouse ESCs and its deletion also leads to decreased proliferation [24]. Interestingly, this proliferative defect could be rescued by over-expression of a membrane bound form of the catalytic PI3K subunit p110 α , providing a further link between PI3Ks and mESC proliferation [24]. Additional evidence to support a role for PI3K signaling in ESC proliferation comes from the finding that deletion of mammalian target of rapamycin (mTOR), a downstream target of the PI3K signaling pathway, leads to an early embryonic lethality in mice and an inability of mTOR null mESCs to proliferate; similar results are observed

upon inhibition of mTOR with Rapamycin [25]. Despite these reports, the molecular mechanisms that underpin the involvement of PI3Ks in regulation of ESC proliferation are unresolved. One possibility is that PI3K-dependent signals contribute to promoting the constitutive activity of cyclinE:Cdk2 complexes in mESCs. In somatic cells PI3K signalling inhibits the activity of the transcription factor FOXO3. In ESCs this could limit expression of p27^{Kip1} meaning cyclinE:cdk2 activity would be largely unchecked. In addition, PI3K-mediated inhibition of GSK-3 activity would prevent the phosphorylation-dependent ubiquitination and degradation of cyclinE, which again could contribute to the maintenance of high constitutive levels of cyclinE:cdk2 complexes that play a role in driving ESC proliferation.

PI3Ks and regulation of mESC fate Our work, alongside that of others, has also implicated PI3K-dependent signaling in control of mouse ESC self-renewal. We were the first to suggest a role for PI3K signaling in maintenance of mESC pluripotency, demonstrating that doses of LY294002 close to its IC₅₀ value of 5 μ M reduced the ability of murine ESCs to retain pluripotency [26]. Other groups have demonstrated that an activated form of Akt, one of the key downstream targets of PI3K signaling, promotes self-renewal independently of LIF [27]. Akt was also identified in an RNAi screen as a regulator of mESC pluripotency [28]. Relatively few studies have examined the role of PI3Ks in the regulation of human ESC fate. PI3K signaling has been implicated in neurotrophin-mediated survival of hESCs [29] as well as maintenance of pluripotency [30], although these studies were limited to the use of LY294002.

We have recently identified genes regulated by PI3K-dependent signaling in murine ESCs, among which are a number of known regulators of pluripotency including Nanog, Klf4, Tbx3, Tcf1 and Esrrb [31, 32]. It has subsequently been suggested that PI3K signaling controls Nanog expression via effects on Tbx3 [33], results entirely consistent with our own observations. Our transcriptome analyses also revealed potential novel regulators of pluripotency including the tyrosine phosphatase Shp1 and members of Zscan4 family of Zinc finger proteins [32]. We demonstrated that Zscan4c plays a role in maintaining ESC pluripotency [32], while more recently it has been reported that the Zscan4 family plays a key role in telomere elongation and genome stability in murine ESCs [34]. These two findings are not incompatible as failure to maintain a stable genome would likely result either in differentiation or apoptosis.

Given the fact that PI3Ks have been implicated in regulation of both ESC proliferation and pluripotency, we hypothesized that particular isoforms of class I_A PI3Ks may couple to distinct functional responses in mESCs. siRNA-mediated knock-down or small molecule-based inhibition of the p110 β PI3K isoform (with TGX-121 or 221) led to a decrease in mESC self-renewal,

suggesting p110 β couples primarily to maintenance of pluripotency. In contrast, siRNA-induced knock-down or inhibition of the p110 α catalytic isoform (with PIK-75 or 15e) reduced proliferation, without significantly affecting pluripotency [35]. Interestingly, we observed that simultaneous inhibition of p110 α and p110 β reduced self-renewal further, leading us to propose that there may be cross-talk between PI3K isoforms in murine ESCs [35]. These observations led us to test whether inhibition of other signaling molecules, also implicated in control of the proliferation of mouse ESCs, could also potentiate the effects p110 β inhibition. We found that sub-optimal doses of Rapamycin, used in conjunction with TGX-121, resulted in a greater loss of self-renewal than inhibition of p110 β alone (Figure 1). Based on our observations it is tempting to propose that PI3K signaling plays a role in integrating the signals that maintain pluripotency and those that regulate mESC proliferation (for model see Figure 2). Clearly further studies will be required to rigorously test this hypothesis.

Glycogen synthase kinase-3 and regulation of ESC fate

GSK-3 is involved in a wide range of physiological processes including development, proliferation and metabolism [36]. In mouse and man two isoforms of GSK-3 exist (α and β) which, although highly homologous, play non-redundant roles [37]. Initial reports relating to the role of GSK-3 in ESCs were conflicting. Ding *et al.*, reported that a kinase inhibitor, termed TWS119, enhanced neuronal differentiation of mouse ES and EC cells [38], the primary target of which was reported to be GSK-3 β . In contrast, Sato *et al.*, using a chemically distinct GSK-3 inhibitor termed BIO, reported that treatment of mouse or human ESCs with BIO enhanced self-renewal and pluripotency [39]. This latter report proposed that BIO was acting as a Wnt mimetic, via inhibition of GSK-3 and led to the suggestion that Wnt signaling played a role in maintenance of ESC pluripotency, a proposal generating much discussion and still not completely resolved [40]. One aspect not considered by Sato and co-authors was the fact that GSK-3 can be regulated by many different signaling pathways [36]. In particular, in response to growth factor stimulation PI3K signalling inactivates GSK-3 via Akt-mediated phosphorylation of Ser9/20 [36]. We sought to clarify the role of GSK-3 in ESC self-renewal and used a chemical genetic approach to achieve this [41]. Our results demonstrated that in the presence of LIF and serum, inhibition of GSK-3 activity (with a series of bis-indolylmaleimides) enhanced mouse ESC self-renewal and in comparison with BIO and TWS119, our GSK-3 inhibitors showed greater selectivity [41]. Interestingly, deletion of all 4 GSK-3 alleles from mouse ESCs cells [42] phenocopied the effect of our GSK-3 inhibitors, providing clear evidence that inhibition of GSK-3 enhances mouse ESC self-renewal. While our studies were nearing completion, Austin Smith's team from Cambridge described the 'Ground state' of mouse ESC pluripotency [40, 43]. Their studies demonstrated that pluripotency of mouse ESCs could be maintained in the absence of exogenous growth factors

using a minimal medium as long as inhibitors of MEK and GSK-3 were present ('2i' conditions). Inhibition of MEK signaling blocks the differentiation inducing effects of autonomously secreted FGF4 [44, 45], but cells cultured with MEK inhibitors alone grew poorly. Addition of GSK-3 inhibitors improved clonal propagation and cell growth, leading to the suggestion that GSK-3 inhibition was required under these very defined conditions to promote cell metabolism and proliferation [40, 43], which in turn is consistent with a role for PI3K signaling in controlling mESC proliferation. In contrast, we had conducted our studies into GSK-3 action in ESCs in a complete medium containing serum and under these conditions inhibition of GSK-3 does not consistently alter ESC proliferation (Sanchez-Ripoll and Welham unpublished data). Therefore, it may be that the differential roles attributed to GSK-3 in defined versus serum-containing media reflect the involvement of GSK-3 in a wide array of cellular responses [36] (see Figure 2) and depending on the cellular context, i.e. the composition of the extracellular environment, particular roles take on greater importance.

More recently we have exploited our newly developed selective GSK-3 inhibitors to re-evaluate the role of GSK-3 in human ESCs. Despite the previous report that BIO can maintain human ESCs in a pluripotent state [39], we have found that our GSK-3 inhibitors do just the opposite and induce differentiation towards mesendodermal lineages (Bone et al., submitted). These observations reflect the findings that treatment of human ESCs with Wnts also promotes differentiation [46-48]. Our recent data further highlight the different responses of mouse and human ESCs to the same factors. The notion that mouse and human ESCs are derived from different developmental stages of embryogenesis has been gaining acceptance within the stem cell community and the differential role of GSK-3 signalling that we have demonstrated adds to the growing list of differences documented between mouse and human ESCs.

Summary

It is clear from recent studies that great progress has been made in our understanding of the range of molecular signals regulating pluripotency, proliferation and self-renewal of embryonic stem cells. The challenge now is to decipher how these pathways are integrated such that maintenance of pluripotency and proliferation are coordinately regulated – a challenge that would surely benefit from a systems biology approach?

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Figure Legends

Figure 1. Partial inhibition of mTOR potentiates the loss in self-renewal observed upon inhibition of p110 β . Murine ESCs were incubated with vehicle (DMSO), LY294002 or the p110 β selective inhibitor TGX-121 at the concentrations indicated in the presence or absence of 0.1nM Rapamycin (Rapa). After 4 days colonies were fixed and stained for alkaline phosphatase activity. Data shown are represented as the mean percentage of alkaline phosphatase positive colonies with S.E.M (n=3). Statistical analysis was conducted using AVOVA and Fisher's post hoc test to compare each treatment, where * indicates $p<0.05$ and ** indicates $p<0.01$. The number of colonies formed in each condition was not significantly different.

Figure 2. Model showing the involvement of PI3K and GSK-3-dependent signals in control of mouse ESC behaviour. The key extracellular, intracellular and nuclear factors that regulate mouse ESC proliferation, pluripotency and promotion of differentiation are depicted. PI3K isoforms play a role in regulation of both proliferation and maintenance of pluripotency, while involvement of GSK-3 signalling in these processes may be dependent on the extracellular environment, refer to text for discussion.

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